Thesis for doctoral degree (Ph.D.)
2007

Gene regulation and immune mechanisms in multiple sclerosis experimental models

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Stockholm 2007

Published by Karolinska Institutet. Printed by Laserics Digital Print AB

ISBN 978-91-7357-280-4

ABSTRACT

Our strategy was to fine map and identify, in an unbiased way, genes or small regions that regulate myelin-oligodendrocyte glycoprotein-induced EAE (MOG-EAE). Such genes are important as they can then be tested for a role in MS. We focused on regions on rat chromosome 4 (RN04) and chromosome 15 (RN15), which were known to regulate neuroinflamation and are rich in immune-related genes.

High resolution mapping of the two regions using data obtained from MOG-EAE experiments in the 7th (G7) and 10th (G10) generation of an advanced intercross line between susceptible DA and resistant, but MHC identical, PVG.AV1 strains confirmed their influence on MOG-EAE. Very narrow QTL confidence intervals with restricted sets of genes were demonstrated. Experiments in congenic strains confirmed the QTLs.

Interaction analyses revealed interesting features within RN04. One QTL, *Eae22,* was only identified when epistasis was considered. Other QTLs, *Eae24*, *Eae26* and *Eae28* were linked to susceptibility phenotypes, whereas *Eae24*, *Eae25*, *Eae26* and *Eae27* were linked to severity phenotypes. Anti-MOG antibody levels for IgG1 were linked to *Eae24*. This is the first time that this immunological sub-phenotype has been linked to a locus that affects EAE.

Studies of innate immunity aspects of MOG-EAE showed that induction of EAE requires MyD88-dependent signaling pathways. MyD88 was required for IL-6 and IL-23 expression in mDC and the number of Th17 cells was reduced in MyD88$^{-/-}$ mice. Further, MOG-EAE was exacerbated in mice that lacked TLR4, TLR4 signaling decreased IL-6 and IL-23 production and TLR4$^{-/-}$ mice had a higher frequency of splenic Th17 cells and more sera IL-17. TLR9$^{-/-}$ mice developed more severe MOG-EAE and higher IL-17 levels than WT mice, indicating that TLR9, like TLR4, regulates rather than promotes MOG-EAE.

In conclusion, the fine mapping studies revealed restricted sets of candidate genes that regulate EAE and gene-gene interactions. Also, innate immunity signaling pathways affect EAE. The findings provide a basis for unraveling immune mechanisms in MS.

LIST OF PUBLICATIONS

I. Maja Jagodic*, Monica Marta*, Kristina Becanovic, Jian Rong Sheng, Rita Nohra, Tomas Olsson and Johnny C. Lorentzen * both authors contributed equally **Resolution of a 16.8 Mb autoimmunity-regulating rat chromosome 4 region into multiple encephalomyelitis QTLs and evidence for epistasis.** J Immunol. 2005 Jan 15;174(2):918-24

II. Monica Marta, Kristina Becanovic,Pernilla Stridh, Maja Jagodic, Johan Ockinger, Johnny C Loretzen, Tomas Olsson. **Analyses of a 58 Mb region on rat chromosome four reveal five distinct loci, Eae24-Eae28, and epistatic interactions in experimental autoimmune encephalomyelitis.** Resubmitted to Genetics

III. Jian Rong Sheng, Maja Jagodic, Ingrid Dahlman, Kristina Becanovic, Rita Nohra, Monica Marta, Ellen Iacobaeus, Tomas Olsson and Erik Wallstrom. ***Eae19*, a new locus on rat chromosome 15 regulating experimental autoimmune encephalomyelitis.** Genetics. 2005 May;170(1):283-9

IV. Monica Marta, Åsa Andersson, Magnus Isaksson, Olle Kämpe and Anna Lobell. **Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis.** Resubmitted to Eur J Immunol

CONTENTS

LIST OF ABBREVIATIONS

ACI	A X C 9935 Irish
AD	Alzheimer's disease
AIL	Advanced intercross line
APC	Antigen presenting cell
BN	Brown Norway
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CSF	Cerebro-spinal fluid
DA	Dark Agouti
DM	Diabetes mellitus
EAE	Experimental autoimmune encephalomyelitis
EAN	Experimental autoimmune neuritis
HS	Heterogeneous stock
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LEW	Lewis
LOD	Logarithm of odds
Mb	Megabase
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MT	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary-response protein 88
NMO	Neuromyelitis optica
OIA	Oil induced arthritis
p.i.	Post-immunization
PLP	Proteolipid protein
PRR	Pattern recognition receptor
PVG	Piebald Virol Glaxo
QTL	Quatitative trait locus
RA	Rheumatoid arthritis
rMOG	Recombinant MOG (aminoacids 1-125)
RR	Relapsing-remitting
SC	Spinal cord
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SP	Seconday progressive
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
α-MOG	Anti-MOG

1 INTRODUCTION

1.1 WHAT CAN WE LEARN FROM MONOGENIC DISEASES?

A mutation in a gene may cause disease when the new phenotype disrupts important biological pathway. If the mutation is not lethal at early stages and does not affect reproduction, it may be carried on to the offsprings. Further, if it confers any sort of benefit, it can be maintained in the population. A major contributing gene has been described in over 2000 phenotypes and diseases (OMIM- www.ncbi.nlm.nih.gov/omim/). Thus, there is evidence for a causative gene mutation or variation for many disease phenotypes. The strategies to identify many of these genes are based on the evaluation of large pedigrees with several affected individuals throughout generations with cytogenetic or linkage analysis, which detect co-segregation of markers with the phenotype.

Monogenic diseases are rare diseases that affect some families or isolated individuals, depending on the penetrance of the gene mutation. Many neurological hereditary diseases and syndromes are classified as monogenic diseases. The mutated allele has high penetrance, where most individuals carrying the allele will develop disease, a pattern of family inheritance can be described in familial cases and there is little influence from environmental factors. This means that there are usually rare variants involved in these rare diseases. Nevertheless, the combination of frequency of the allele in the population and frequency of the diseases they cause is variable. For example, in a common condition, deep vein thrombosis, the rare Factor V Leiden allele is involved in a minority of cases; the rare early-onset familial cases of the common Alzheimer's disease (AD) are caused by rare alleles; the same common disease AD is influenced by the much more common ApoE4 allele (Corder EH *et al* 1993) and a comparable situation happens for type 2 diabetes mellitus (T2DM) and PPARgamma Pro12Ala (Li WD *et al* 2000).

It is important to mention that many diseases historically thought to be monogenic are now viewed as oligogenic or even complex. In such cases, the penetrance and expression of a disease allele could be substantially affected by other genetic and environmental modifiers. Even the most well described diseases have alternative clinical courses, response to treatments and outcomes that cannot be accounted for by environment only. Therefore, the complexity involved even in monogenic diseases must be appreciated (Badano and Katsanis 2002, Soares ML *et al* 2005).

Identical phenotypes may be caused by different genes, which is a risk for the studies where the causative gene mutation is sought in unrelated people. Several hereditary neuropathies and ataxias have similar main clinical features but have different genetic causes. Less common are cases where, different types of mutations in the same gene can cause differences in severity of the phenotype.

Genes involved in monogenic diseases have been easier to identify probably due to a stronger effect than those involved in complex diseases. Identification of the mutated gene is still one important step to solve the mechanism of disease.

1.2 COMPLEX TRAITS AND COMPLEX DISEASES ARE UBIQUITOUS.

In contrast to complex diseases, most traits or phenotypes are regulated by several genes working in concert and are modified by environmental influences. Complex mammalian traits include height, weight, behavior, metabolic features and other measurable characteristics. Complex diseases are characterized by traits that are out of the normal range to meet pathologic criteria and are similarly regulated by many genes and modified by the environment. This is the case for hypertension, most cancers, psychiatric diseases, inflammatory diseases like atherosclerosis, systemic lupus erythematosus (SLE) and multiple sclerosis (MS). There seems to be a threshold above which the combination of alleles together with environmental factors causes an individual to become diseased. It is not yet clear whether there is an “unfortunate” combination of normal alleles, or gene polymorphisms, or a combination of abnormal variants, gene mutations, in a more or less protective background. The interplay between genes and environment affects

not only the susceptibility or risk of developing the disease, but also its course, severity and response to treatment. Most individuals are likely to be affected by a complex disease at some point during their lifespan.

This genetic share can be expected when there is familial aggregation of cases and high incidence in some ethnic populations compared with other populations, and incidence is independent of geographic location. Evidence of risk heritability is measured by the degree of familial aggregation and is determined by the ratio of prevalence in relatives versus prevalence in the population. Even when family clustering of cases is observed, this alone does not reveal the inheritance pattern. The fact that some diseases have familial forms does not grant them a complex etiology, but the familial aggregation must be assessed in the more isolated cases. The environmental triggers are sometimes recognized, such as cigarette smoking in some cancers and rheumatoid arthritis (RA) or diet in T2DM, but their contribution is difficult to measure. Even if a lot of sensible life-style changes can be recommended to modify or eliminate the environmental triggers, knowledge of the genetic etiology will still be essential to understand pathogenesis and response to therapy. Therefore, prior to extensive genetic studies there sould be a confirmation of the genetic component of the etiology.

Most techniques and statistical strategies used to study complex diseases were developed to study monogenic diseases. When investigating which genes are involved in complex diseases it is difficult to isolate the effect of one gene amid the “noise” of other genetic and environmental contributors. Most gene variants initially identified in complex diseases were causative mutations or polymorphisms in the rare forms of those diseases that showed mendelian inheritance. They were not always relevant for the majority of the people affected with a similar phenotype. This is the case for *BRCA1* and *BRCA2* in breast cancer and the familial early onset forms of Alzheimer’s and Parkinson’s disease.

Much progress has recently been achieved with the identification of gene polymorphisms that influence complex diseases and the pathologic mechanisms those genes expose. Much more needs to be understood about how the combinations of polymorphisms disturb the balance from health and lead to disease and what can be done to restore that balance.

1.3 THE GENETIC APPROACH IS CENTRAL TO FIND GENES IN DISEASES WITH COMPLEX ETIOLOGY.

Despite all the challenges, finding genes that are responsible for both monogenic and complex diseases is a robust route to understand disease mechanisms and possible treatments or even cure. As mentioned, the first step in this approach is to establish that the disease has a clear **genetic etiology**.

Genetic markers are DNA sequence stretches with a known location that allow the distinction of individuals when there is a variation at the position. These markers can be tested for their segregation with disease or phenotypes (Botstein D *et al* 1980) and highlight genomic regions linked to disease. Originally restriction fragment length polymorphisms were used, later microsatellites were the choice. Nowadays, the high frequency and wide distribution of single nucleotide polymorphisms (**SNPs**) and the many typing techniques available makes SNPs the first choice. The International HapMap consortium project (HapMap 2003) typed 1 million SNPs and aims at 4.6 million SNPs in 270 individuals from Chinese Han, Japanese, European and African Yoruba descents in order to characterize the world population and to evaluate which SNPs were in linkage disequilibrium, were co-inherited and could represent their region. It is often necessary to resequence a number of individuals from a particular study population to ensure that the allele variations are acurately represented at comparable frequencies in the study population and the "representative" populations within the HapMap project. With the sequencing of the rat and mouse genomes, polymorphisms became easy to locate and assay (Mouse Genome Sequencing Consortium 2002 and Rat Genome Sequencing Project Consortium 2004).

DNA variations or polymorphisms may be used just as location markers or they may constitute disease causative variations. It is important to detect the phenotypic effects that the polymorphisms have by themselves, but also the effects they have on biological networks that drive the disease (Schadt EE 2006). The possible models encompass a DNA variation that causes a distinctive expression that affects disease susceptibility (causal model) or vice-versa (reactive model) or a DNA variation that causes independent distinctive expression and phenotype (independent model). One must bear in mind that evidence for linkage or association is not proof of genetic causality.

Nevertheless, when the identified variant is under selective pressure and is functional there is a higher chance that it contributes to disease.

Linkage analysis of families is a search for co-segregation of a phenotype with a marker or markers in linkage with the causative variation and linked in the family. This process highlights a genomic region linked to disease and allows further positional cloning and direct testing (Lander and Botstein 1986). **Association studies** use linkage disequilibrium between the markers and the causative variation in a population, over many generations, and aims to establish frequency differences in cohorts of patients and controls.

Studies may be based on an educated guess, where a **candidate** is chosen because of previous knowledge and is then tested in an association study. On the contrary, the **hypothesis-free** studies are unbiased and identify both known and "expected" genes or totally unknown genes or even intergene regions. These are most often genome-wide linkage scans which look for increased allele sharing at marker loci among individuals who show similar phenotypes (e.g. sib-pairs analysis) and more recently genome wide association studies.

The **quantitative trait locus/loci (QTL) analysis** is a means of finding novel genes or genomic loci that regulate complex traits. Individuals are characterized by diverse quantitative measurements and the analysis investigates the degree to which this variation can be explained by genetic and environmental components. The regions identified are usually large and contain a substantial number of genes. The fine-mapping to reduce size of QTL intervals can be achieved using populations with more genetic diversity, haplotype analysis, focused association studies or comparative genomics (DiPetrillo K *et al* 2005).

1.4 MULTIPLE SCLEROSIS IS A DISEASE WITH COMPLEX GENETICS.

Multiple sclerosis (MS) is a disease characterized by chronic inflammation, demyelination and degeneration in the central nervous system (CNS). According to the consensus criteria (Polman CH *et al* 2005, McDonald WI *et al* 2001, Poser CM *et al* 1983) the clinic features of neurological deficits should be typical, lesions should occur in different locations at different time points and other possible causes must be excluded. Clinically, MS may have a relapsing

or progressive course eventually leading to severe disability. The histopathological features have 4 patterns: i. active demyelination with relative preservation of oligodendrocytes and T cell and macrophage infiltrates; ii. similar as above but with immunoglobulin and complement 9 antigen deposition; iii. pronounced loss of oligodendrocytes, and the demyelination and cellular infiltrates did not surround inflamed venules; iiii. non-apoptotic oligodendrocyte death and demyelination with cellular infiltrates (Lucchinetti CF *et al* 2000). These histological patterns do not correlate with clinical patterns except for the rare last pattern which can be found in patients with primary progressive MS. Many questions regarding susceptibility, timing of relapses, course or even response to treatment are unanswered and probably depend on genetic characteristics. The lack of surrogate diagnostic and disease course markers calls for high clinical accuracy, namely in recruitment of patients for genetic studies. The exception is a severe form of MS, neuromyelitis optica (NMO) or Devic's syndrome, which affects optic nerves and spinal cord with little recovery or response to current MS therapy. Recently, a serum autoantibody (NMO-IgG) binding to aquaporin 4 (AQP4) water channels in CNS was identified in cerebro-spinal fluid and sera of NMO patients (Lennon VA *et al* 2005), which allows early discrimination from "classic" MS or other inflammatory CNS diseases and consequential therapeutic choices.

The genetic component of MS and recurrence risk were extensively evaluated by the Canadian Collaborative Study Group and others in studies with half-siblings (Sadovnick AD *et al* 1996), adoptees (Ebers GC *et al* 1995), spouses (Ebers GC *et al* 2000) and the results appear to confirm that genetic, and not environmental, factors are primarily responsible for the familial clustering of cases. The familial recurrence rate is about 15%. Recurrence in monozygotic twins is around 35%. The age-adjusted risk is higher for siblings (3%), parents (2%), and children (2%) than for second degree and third-degree relatives. The risk for half-siblings is less than for full siblings. Recurrence is higher in the children of conjugal pairs with multiple sclerosis (20%) than when only one of the parents is affected (2%). Conversely, the risk is not increased either for individuals adopted into a family with an affected individual or in the non-biological relatives of adoptees who themselves develop multiple sclerosis. Many population based linkage and association scans have been conducted and the only region that is repeatedly associated with MS is the HLA region. In

particular the DR15 and DQ6 alleles, DRB1*1501 and DQB2*0602, have been a consistent finding across nearly all populations (Oksenberg and Barcellos, 2005, Silva AM *et al* 2007). A major breakthrough in the search for polymorphisms consistently associated with MS happened very recently with the identification of IL-7 receptor α. The results were replicated in several populations (Gregory SG *et al* 2007, Lundmark F *et al* 2007, Hafler DA *et al* 2007). These studies also offer clues on how the gene may be leading to disease, either by altered expression in the cerebro-spinal fluid (CSF) cells compartment or with altered alternative splicing. This gene was among the first candidates to be identified in a mouse cross with MBP peptide-EAE (Sundvall M *et al* 1995). Large Mendelian pedigrees can sometimes elucidate pathological pathways of complex diseases (Cookson MR *et al* 2005), but the rare family described for MS was not informative (Dyment DA *et al* 2007).

Some gene polymorphisms that influence MS

Results replicated	Studies repeated but not replicated
MHC2TA (Swanberg M *et al* 2005, Martinez A *et al* 2007)	ApoEε4 (Burwick RM *et al* 2006, Pinholt M *et al* 2006, Koutsis G *et al* 2007)
IL7R (Teutsch SM *et al* 2003, Zhang Z *et al* 2005, Booth DR *et al* 2005, Gregory SG *et al* 2007, Lundmark F *et al* 2007, Hafler DA *et al* 2007)	CTLA4 (Kantarci OH *et al* 2003, Heggarty S *et al* 2007, Dincic E *et al* 2007, Suppiah V *et al* 2005, Dyment DA *et al* 2002, van Veen T *et al* 2003, Fukazawa T *et al* 2005, Roxburgh RH *et al* 2006, Bagos PG *et al* 2007)
PRKCA (Barton A *et al* 2004, Saarela J *et al* 2006)	CD24 (Wang AL *et al* 2007, Otaegui D *et al* 2006, Goris A *et al* 2006, Zhou Q *et al* 2003)
	PTPN22 (Begovich AB *et al* 2005, Criswell LA *et al* 2005)

There are many reasons for failures to identify and replicate the association of gene polymorphisms in MS. Many studies do not have enough statistical power due to small numbers of recruited individuals. To increase cohort size, the risk that individuals with heterogeneous genetic background are included increases, both in patients and controls groups, and there may be a population stratification caused by inclusion or population bias. Both genotype errors and low efficiency severely hinder statistical power. MS may be difficult to diagnose because of a purely clinical diagnosis, heterogeneous patients and absence of surrogate markers of disease. In addition, the characterization of patients rarely includes quantitative traits (Cardon LR *et al* 2003). Early adolescence exposure to environmental factors that influence the development of MS are detected at

a population level but not at family level (Kennedy J *et al* 2006). Several studies show the influence of vitamin D levels (Munger KL *et al* 2006), sunlight exposure (Islam T *et al* 2007), smoking (Hawkes CH 2007) and infections (Thacker EL *et al* 2006) on development of MS. In general, studies may be difficult to replicate because the ones with stronger effect are published first (winner's curse Risch and Merikangas 1996), different SNPs for the same genes are used, and linkage disequilibrium around candidate genes is typically not considered (Abou-Sleiman PM *et al* 2004). When such problems are addressed, better results can be expected.

1.5 ANIMAL STUDIES ARE AN ALTERNATIVE STRATEGY.

An animal model for MS, experimental autoimmune encephalomyelitis (EAE), is attained with the subcutaneous injection of myelin protein antigens together with adjuvants that promote pro-inflammatory responses in susceptible animals. The antigens, myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) protein or their peptides or a homogenate of spinal cord or myelin, are emulsified with mineral oils (Freund's adjuvant) and inactivated and dried *Mycobacterium tuberculosis* and injected, often with pertussis toxin intraperitoneally. EAE may also be caused by the transfer of $CD4^+$ T cells from EAE affected rodents to healthy susceptible recipients (Ben-Nun and Cohen 1982). The susceptibility to develop EAE signs depends on the inbred strain genetic background, particularly on the MHC region. This was demonstrated for rat EAE three decades ago (Gunther E *et al* 1978), and recently demonstrated for MOG-EAE in the strains we use (Wallstrom E *et al* 1997, Weissert R *et al* 1998) and on the induction protocol. When Dark Agouti (DA) rats are injected with $MOG_{1\text{-}125}$ in emulsion with Freund's adjuvant they develop a disease that mimics MS both pathologically (Storch MK *et al* 1998) and its clinically relapsing course. The rats are evaluated for EAE signs according to a mobility score and the disease is characterized by phenotypes such as onset and duration of EAE signs; mean, maximum and cumulative EAE score. These are not truly quantitative traits but their variation can be measured and used as a quantitative trait in genetic studies. The heterogeneity of the background as well as some environmental influences can be controlled for with inbred strains kept in the same facility. The

different models in mice and rats have characteristics that can be used to address a variety of questions, which also means they may lead to different results.

Rodent model are invaluable for genetic (and immunological and treatment) studies for a number of reasons. Assuming that the homologous human genes or pathways regulate the corresponding human traits, we pursue rodent QTL genes. There are striking similarities between rodents and humans on biological functions such as development, immunology and cell division, and probably evolutionarily conserved disease pathways too. Comparative genomics identifies regions of chromosomal synteny in QTLs that are concordant across species and relevant for disease (Serrano-Fernández P *et al* 2005). Animal models have identified some genes that have influence on the equivalent human disease (*Tnfsf4*- OX40 ligand- in atherosclerosis, *Ctla4* in type I diabetes and FCGR3B in systemic autoimmunity). Cross-species sequence comparison assists in locating gene regulatory elements (Loots GG *et al* 2000). These examples show that at least some mechanisms of disease can be addressed through animal models.

As the differences in susceptibility to EAE depend on genetic background, F2 and back-crosses of susceptible and resistant rodents allow linkage to large genomic regions. The number of siblings with known parental genotypes that can be tested in each generation is critical in the statistical power and is limited mainly by lab resources. Many techniques are used to narrow the confidence intervals obtained in F2 and backcrosses. In our laboratory, one of the most used is the advanced intercross lines (AIL) between EAE susceptible DA and EAE-resistant PVG.AV1, in which generations beyond F2 up to G10 were bred to increase recombination frequency (Iraqi F *et al* 2000, Jagodic M *et al* 2004). In this particular case, to avoid the known strong influence from the MHC region, the parental rats had identical MHC haplotype, AV1. In combined cross analyses, the data from crosses of different inbred strains are analyzed together to find QTLs at the same location, and the susceptibility alleles are supposed to have the same ancestral origin (Li R *et al* 2005, Jagodic M and Olsson T 2006).

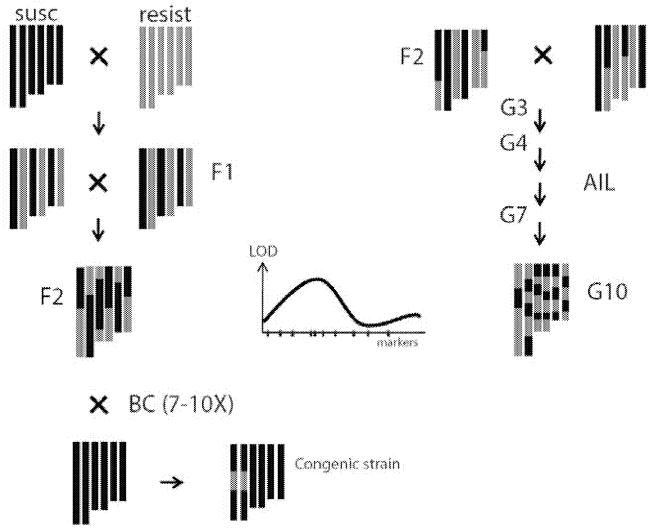


Figure 1. Breeding scheme between susceptible (susc) and resistant (resist) inbred strains to obtain an F2 cross or a backcross (BC) that can also create congenic strains. The intercross between F2 rats with avoidance of brother-sister mating is kept on for several generations to create an advanced intercross line (AIL). Linkage analysis in the data from EAE experiments in F2, BC or AIL cohorts enables mapping of EAE QTLs.

Haplotype analysis can decrease confidence intervals even more dramatically, by identifying high priority regions within a QTL interval that are likely to contain the causal polymorphism; that is, regions that are shared by susceptible versus resistant strains (Swanberg M *et al* 2005). The heterogeneous stock (HS) of rats, a population derived from several known inbred founder strains and kept heterozygous by random mating, is another way to increase genetic variation (Tabakoff and Culp 1984, Talbot CJ *et al* 1999, Waldar W *et al* 2006).

Congenic strains are particularly useful to confirm and fine-map QTLs (eg. Becanovic K *et al* 2003a, Sheng JR *et al* 2005), to study molecular mechanisms and pharmacogenomics. They are characterized by a variable size segment of genome from a donor strain in a recipient strain background. This is achieved by intercrossing the parental strains and subsequent repeated backcrossing. The amount of the donor genome in the congenic strain background depends on the number of backcross generations. Congenic

strains with overlapping donor genome stretches may be tested simultaneously to determine a necessary interval for effect, a technique sometimes called congenic mapping. Further crosses to increase recombinations and progeny testing also helps to narrow QTLs. Polymorphic DNA markers allow accelerated creation of congenic strains by marker-assisted breeding ('speed' congenics) (Wakeland E *et al* 1997).

Spontaneous gene mutant rodents can be compared to the inbred strain they derive from after induction of EAE to test the effect of the mutation. Chemically-induced mutations, usually obtained with *N*-ethyl-*N*-nitrosourea (ENU), can provide novel mutant mice (Rathkolb B *et al* 2000) or rats (Ohgaki H *et al* 1993). Engineered mice, such as transgenic and knock-out mice, created from homologous recombination in embryonic stem cells from 129 or C57BL/6 mouse strain (Seong E *et al* 2004) provide the tools to manipulate mammalian genes *in vivo*. Manipulation of the mouse genome requires previous knowledge of gene structure, which is now provided by the almost complete mouse genome. Unlike classical phenotype-driven strategies (for example, positional cloning of a spontaneous mutation with a relevant phenotype), the forward genetics approach is based on searching for a phenotype after a gene is mutated (Phillips TJ *et al* 2002). Conversely, after random mutations and phenotype selection it is possible to positionally clone the responsible gene. Beutler and co-workers disclosed the function of most TLR pathway molecules using the same phenotype driven unbiased mapping technique. In particular, a spontaneous mutation in the *Lps* locus was positionally cloned and happened to be similar to Tlr4. They then proceeded to look for tumor necrosis factor (TNF) synthesis and activity as the phenotypes to identify the genes in the randomly mutated TLR pathway and found the genomic locations of several of the TLRs and signaling molecules (Poltorak A *et al* 1998, Beutler B 2005).

Gene expression levels are quantitative traits and expression databases allow the identification of genes that are differentially expressed among inbred strains. Microarray data from tissues of F2 animals enables identification of expression QTLs (eQTLs) that regulate transcript levels. eQTLs that co-localize with the gene in which transcription is being regulated are referred to as *cis* eQTLs (in contrast to trans-eQTLs). When they co-localize with QTLs for a clinical trait, they can greatly facilitate the search for causal genes. Moreover,

microarrays and eQTLs can identify pathways, because one can interrogate many transcripts simultaneously. Integrating genetic and gene-expression data on a global basis might allow the identification of regulatory genes that have a strong influence on a clinical trait (Carlborg O *et al* 2005).

These strategies are powerful approaches to the identification of regions and genes and elucidation of their function. Much of what we know about human immunology arose from rodent studies, and the principles appear to hold true although evolutionary selection of human and rodents must differ.

1.6 WHAT HAVE GENETIC STUDIES IN RAT EAE TAUGHT US SO FAR?

Over the years, our laboratory has studied animal models of MS and has gathered considerable knowledge on the immunological and genetic control of EAE.

The DA rats are susceptible to EAE induction with spinal cord homogenates (SC) in incomplete Freund's adjuvant (IFA) or complete FA (CFA), MOG_{1-125} in CFA or IFA and MOG peptides in CFA. The relapsing-remitting (RR) course of EAE displayed progressive worsening followed by amelioration of signs (Lorentzen JC *et al* 1995). Major influences on EAE map to the MHC region (Mustafa M *et al* 1994, Wallström E *et al* 1997, Weissert R *et al* 1998). When the DA MHC class II haplotype $RT1^{a}$, also referred to as $RT1^{av1}$ or 1AV1 or AV1, is introduced into different strains it confers a variable degree of susceptibility to EAE (reviewed in Becanovic K *et al* 2004). The Lewis (LEW).AV1 rats also have a RR EAE course after injection with SC and MOG_{1-125} in CFA (Adelmann M *et al* 1995). The PVG.$RT1^{c}$ rats display a brief and mild monophasic EAE after MBP_{63-88} in CFA whereas PVG. $RT1^{av1}$ rats display a more severe monophasic disease. In contrast to the inflammatory and demyelinated lesions in MOG-EAE, MBP induced EAE is a T cell mediated disease only and does not have demyelination. PVG.$RT1^{av1}$ rats are relatively resistant to MOG_{1-125} even in CFA, but develop severe EAE signs once affected, after immunization with high dose of the antigen. In an analogous way to MS, MHC haplotypes determine the degree of susceptibility. In rats, its influence has been determined also for the clinical course, the recruitment of encephalitogenic cells to CNS and CNS pathology in EAE models. But in order

to induce EAE it is necessary to have not only the correct MHC haplotype but also a permissive background.

To map EAE QTLs, experiments have been conducted on four F2 crosses between susceptible and resistant rat strains in our laboratory. (DA X BN) F2 rats were injected with SC in CFA and showed linkage to chromosome 9 (*Eae4*) (Dahlman I *et al* 1999a) and suggestive linkage to a locus in chromosomes 7, 12 (overlapping *Eae5* and *Pia4*) and 15 (later *Eae19*). (DA X ACI) F2 rats were injected with MOG_{1-125} in IFA and showed linkage to loci in chromosomes 10 (*Eae12*), 12 (*Eae13*), 13 (*Eae14*) and 18 (*Eae15*) (Dahlman I *et al* 1999b). (LEW.AV1 X PVG.AV1) F2 rats were injected with MOG in IFA and showed linkage to loci in chromosomes 8 (*Eae16*) and 13 (*Eae17*) (Becanovic K *et al* 2003b). In a (DA X PVG.AV1) F2, 70 rats were injected with MOG in IFA and linkage searched at locations of five experimental arthritis QTLs: linkage was detected to chromosome 4 markers located on *Cia3* and *Oia2* (Dahlman I *et al* 1998). In other laboratories, two other F2 crosses with different strains combinations have identified EAE QTLs. (LEW X BN) F2 rats were injected with myelin in CFA and showed linkage to loci in chromosomes 4 (*Eae2*) and 10 (*Eae3*) (Roth MP *et al* 1999). (DA X E3) F2 rats were injected with SC in CFA and showed linkage to loci in chromosomes 1 (*Eae1*, *Eae6* and *Eae7*), 12 (*Eae5*), 19 (*Eae8*), 6 (*Eae9*), 14 (*Eae10*) and 4 (*Eae11*) (Bergsteinsdottir *et al* 2000). The loci detected on chromosome 4, *Eae2* and *Eae11*, are both centromeric and may overlap, but are at a distinct location from the ones detected in the (DA X PVG.AV1) F2 cross. The loci detected on chromosome 10, *Eae3* and *Eae18*, are also centromeric and may also overlap, but are distinct from *Eae12* detected in (DA X ACI) F2 cross. The loci detected on chromosome 12, *Eae5* and *Eae13*, possibly overlap. Each EAE QTL is characterized by the strain combination and the induction protocol, so that the effect of alleles at a particular physical location of one strain may differ depending on such factors. The multiplicity of non-MHC EAE QTLs reflects the polygeneity and genetic heterogeneity in EAE that is believed to also occur in MS. Further, the identification of such QTLs over different chromosomes in different studies is a result of their weak effects.

The next step was to narrow QTL confidence intervals and to confirm their relevance. Congenic strains, which hold an isolated QTL allele in a genetically different background, can help to achieve both objectives. Our laboratory

established an advanced intercross line (AIL) between DA and PVG.AV1 and ran MOG-EAE experiments in the 7th and 10th generations that proved fundamental to narrow QTL intervals. One of the regions identified on chromosome 10 in the (DA X ACI) F2 cross is also linked to oil induced arthritis (OIA) (Lorentzen JC *et al* 1998). The ACI alleles, which covered around 60 Mb confidence interval on a DA background, conferred protection with lower incidence of EAE confirmed histopathologically and defined *Eae18* (Jagodic M *et al* 2001). The region of interest was reduced with congenic recombinant strains derived from the above and the fine-mapping of the region in the G7 AIL data separated two distinct QTLs, *Eae18a* and *Eae18b* (Jagodic M *et al* 2004). Further linkage analysis in the G10 AIL data and the use of an *in-silico* strategy that combines data from humans and animal models studies defined an overlapping 1.06 Mb region that contains 13 genes (Öckinger J *et al* 2006). *Eae13*, in a combination of DA and ACI alleles, and *Eae5*, in a combination of DA and E3 alleles, overlap with *Pia4* location on chromosome 12. Positional cloning of *Pia4* led to the identification of a naturally occurring polymorphism in *Ncf1* that regulates arthritis severity in rats (Olofsson P *et al* 2003). Fine-mapping of this region in the G10 AIL MOG-EAE data narrowed the interval to 1.4 Mb that includes *Ncf1*. Polymorphisms in the regulatory region and exons were detected in DA X PVG.AV1 strain combination too. Expression analysis (quantitative real-time PCR) of all the genes in a panel of naïve and MOG-immunized rat tissues detected consistent expression differences between DA and PVG.AV1 in *Cldn4* but not in *Ncf1* (Becanovic K *et al* 2006). In chromosome 4, a region that overlaps experimental arthritis QTLs *Aia2* and *Pia5* showed a degree of linkage to MOG-EAE survival and levels of serum anti-MOG IgG2c antibodies in the DA X PVG.AV1 cross. Several recombinant congenic strains with PVG alleles over the 70cM region on a DA background were created and tested for models of experimental arthritis, such as rat type II collagen in IFA (CIA), pristane induced arthritis (PIA), *Mycobacterium tuberculosis* adjuvant-induced arthritis, squalene-induced arthritis (SIA) and oil-induced arthritis (OIA) (Bäckdahl L *et al* 2003, Ribbhammar U *et al* 2003). The effect of MOG-EAE was tested in the same recombinant congenic strains which made comparisons between the two models possible. The PVG alleles in the DA.PVG–*chrom4.R1* congenic strain were protective to MOG-EAE while they had no effect on CIA or PIA. Paradoxically, the PVG alleles in DA.PVG–

chrom4.R3 conferred protection in experimental arthritis but caused more severe phenotype in MOG-EAE (Becanovic K *et al* 2003a). In this thesis, the divergent effects of PVG alleles on chromosome 4 congenics and the influence from chromosome 15 observed in the (DA X BN) F2 cross were further studied.

Additive effects and epistatic interactions were suggested by some of the accumulated data. The (DA X BN) F2 cross was not controlled for MHC and an additive effect at that locus was noted. Population stratification analysis showed that rats homozygous for AV1 had a higher EAE incidence effect on *Eae4* than the rats that were heterozygous for those MHC alleles. The observation that the larger chromosome 4 congenic strain had no effect on EAE while the smaller non-overlapping R1 and R3 were protected or had aggravated EAE is later investigated as a sign of epistatic interaction (Paper I) The (LEW.AV1 X PVG.AV1) F2 was a reciprocal cross that detected parent of origin effect at *Eae17*. This effect could be due to mitochondrial influence or to epigenetic mechanisms.

1.7 ARE EPISTATIC INTERACTIONS NEGLECTED OR OVER-RATED?

Interactions between genes or between genes and the environment substantially contribute to variation in complex traits. In early reports, each gene was studied according to the major individual effect on the phenotype, so most studies ignore interactions and detect only QTLs with large effects. This has been proposed to be one of the reasons for difficulties in the replication of genetic studies. Nevertheless, there are many reports that address interaction effects without designating them as interactions. For example, the phenotype effects of a knockout or transgene that differ between mouse strain backgrounds constitute evidence for the existence of epistatic interactions. Variable penetrance of the effect of a gene can be caused by interactions with an external trigger or with a modifier gene effect (Alper CA *et al* 2000). The fact that individual loci will remain undetected and the estimated effects of each QTL will be inaccurate emphasizes the importance of interactions.

Interactions may show either be additive or epistatic. Interactions have additive effects when the final result depends on the sum of the effects that drive the phenotype in the same direction (e.g. Weedon MN *et al* 2006). Alternatively, interactions have epistatic effects when the phenotype of a given

genotype is not predicted by the sum of its known single-locus effects. Epistasis implies that the effect of one particular genotype on phenotype is modified by one or several genes that assort independently. Epistasis can occur at the genomic level, where one gene could code for a protein preventing transcription of the other gene. Alternatively, the effect can occur at the phenotypic level, when the effect of one gene "overcomes" the phenotype of a different gene. Two-locus epistatic interactions can be either synergistic (positive) or antagonistic (negative).

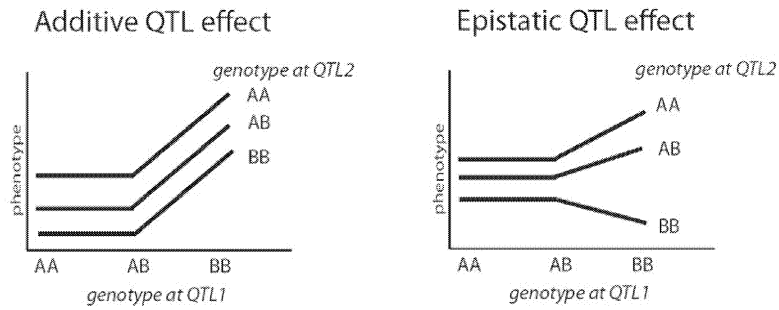


Figure 2. The effect of additive QTLs is changed by each allele's effect whereas the effect of epistatic QTLs in changed by more or less than each allele's effect.

Epistatic QTL mapping methods simultaneously consider the mean effects of multi-locus (usually two, maybe three) genotypes on the phenotype. Epistasis can be detected between pairs of QTLs where one or both have been detected by their individual effect or when none would have been detected (minor individual effect but novel epistatic loci) (Paper I). Although some genes with epistatic effects will be discovered because they have both direct and indirect (epistatic) effects, others would only be discovered by experiments intentionally designed for this purpose. But it is possible to simultaneously map QTLs using an epistatic model detecting loci that mainly affect the quantitative traits through epistatic interaction with another locus, in addition to those QTLs that are detected through their individual effect. One of the major problems with studies of epistasis is the inadequate statistical power of most cohorts. The study of gene x gene interactions requires much larger sample sizes than studies of direct effects. Nevertheless, there are examples of studies that

address the complex interactions among MHC haplotypes in MS and in type 1 diabetes mellitus (Dyment DA *et al* 2005, Koeleman BP *et al* 2004) and that detect significant epistasis between SNPs from genes in the inflammatory pathway (Motsinger AA *et al* 2007). A practical approach to find genes involved in epistasis is to use animal models because the genotypes are known and reproducible. In our studies of MOG-EAE in the AIL, we assess the occurrence of epistasis and have thus identified it in two regions in rat chromosome 4 (Paper 1 and Paper 2), one of which had not been detected in an isolated way. In arthritis models in mice, *Cia5* and *Eae2* were dissected with the definition of epistatic loci in a partial advanced intercross (Johannesson M *et al* 2005, Karlsson J *et al* 2005).

In quantitative genetics, epistasis relates to the improvement in the prediction of phenotypic variation from simultaneously considering multiple-locus genotypes, relative to the prediction from the sum of single locus genotypes. But these estimates of epistasis do not have a direct relationship with the biological mechanisms. Therefore, it is necessary to evaluate all combinations of loci using post-mapping analyses to evaluate biological significance. To accomplish this, we plotted the combinations of two loci genotypes on phenotype patterns for epistatic pairs. Information on gene regulatory networks can also help to analyze biological significance of interactions to the same extent that understanding epistatic interactions will help to unravel new networks. Some of this information is already available in on-line databases (Omholt SW *et al* 2000). Studying genetic interactions can reveal gene function, the nature of the mutations, functional redundancy, and protein interactions. Because protein complexes are responsible for most biological functions, genetic interactions are a powerful tool, as seen in mouse lupus (Bolland *et al* 2002, Wu X *et al* 2002).

To summarize, the analysis of epistasis, or gene-gene interactions, is of particular importance for revealing the molecular mechanisms of complex human diseases. Multiple genes, each of which has a moderate effect, might interact and produce a complex phenotypic trait (Schadt EE 2006, Liang KH *et al* 2007). Importantly, one must remember that interactions may involve genes only, genes and environment, genes and protein or protein networks.

1.8 NEW CONCEPTS ON THE IMMUNOPATHOLOGY OF EAE

According to Janeway, the ability of the immune system to discriminate between “self” and “non-self” enables the generation and maintenance of tolerance (Janeway CA Jr 1989). Furthermore, Matzinger proposes that the immune system’s primary task is to sense ”danger” both from outside and inside the body (Matzinger P 1994). These theories are brought together with the concept of pattern recognition receptors (PRR) on immune cells that recognize conserved molecular patterns (pathogen-associated molecular patterns or PAMPs). There are three categories of PRRs: soluble extracellular receptors like complements, membrane-associated receptors like Toll-like receptors (TLRs) and intracellular receptors like nucleotide-binding oligomerisation domain (NOD) proteins.

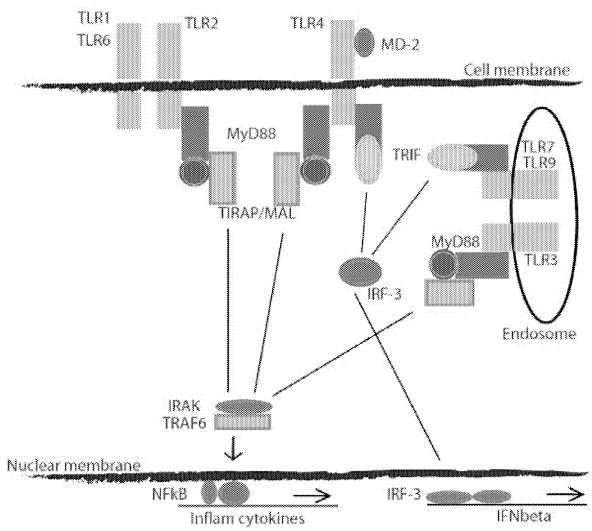


Figure 3. Toll-like receptors signaling pathways

Toll-like receptors are expressed on the innate immune system cell surfaces (TLR1, 2, 4, 6, and 11) or on intracellular endossomal membranes (TLR3, 7, 9) (Figure 3). After binding of ligands to LRR (leucine rich receptors) domains of TLRs, TLRs may join other proteins to construct the heteromeric receptor complex. Consequently, the signalling pathways are initiated by interaction of TIR domains of TLRs with adaptor molecules. Four adaptor

proteins, MyD88 (myeloid differentiation primary-response protein 88), TIRAP, TRIF (TIR-domain-containing adaptor molecule inducing interferon-β) and TRAM, have been identified as important transducers of TLR signals. These adaptors aid the activation of different protein kinases that in turn activate the transcription factor nuclear factor-kappaB (NF-κB) and various mitogen-associated protein kinases. Other molecules such as interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK4, tumour necrosis factor-receptor-associated factor 6 (TRAF6), and inhibitor of NF-κB kinase (IKK) complex are also involved in these pathways. These pathways lead to activation of NF-κB and/or interferon regulatory factor 3 (IRF3) and various mitogen-associated protein kinases. NF-κB controls the production of cytokines and proinflammatory products, like interleukins (IL-1, IL-6, IL-8) and tumour necrosis factor (TNF); meanwhile, IRF3 controls the production of interferon type I (IFN-α, IFN-β). It is possible that TLR activation can by-pass tolerance and can induce autoimmunity, overcoming tight regulation by SOCS (suppressors of cytokine stimulation) proteins and regulatory cytokines.

NODs are a family of intracellular PRRs that include NOD1 and NOD2-LRR proteins. They help the immune system to recognize PAMPs, such as bacterial peptidoglycans. After binding the ligand to the LRD domain of NODs, the signalling pathway continues with the interaction of EBD domains (or CARD) of NODs with receptor-interacting serine/threonine kinase (RICK). This pathway leads to activation of caspases and NF-κB, which controls the production of cytokines and proinflammatory products (Rezaei N 2006).

One of the proposed roles for TLRs in autoimmune disease is to activate antigen-presenting cells (APC) in the target organ. New evidence supports the influence of TLRs on autoimmune diseases, such as the characterization of TLR4 polymorphisms linked or associated with inflammatory bowel disease (Franchimont D *et al* 2004, De Jager PL *et al* 2007) or the role of TLR9 in SLE (Tao K *et al* 2007) among others. There is a broad and regulated expression of TLRs in human CNS. For example, TLR3 and TLR4 are expressed in regions of inflammation in MS lesions, but expression is also present in non-inflamed microglia and neurons (Bsibsi M *et al* 2002). The data on TLRs and EAE is still scant. The study by Prinz et al in a MOG-peptide mouse model showed that MyD88 is necessary for EAE development, which we have corroborated, but

also show that signaling through TLR9 is necessary, which is the opposite of our findings (Prinz M *et al* 2006).

Until recently, the T cell adaptive immunity field held that naïve CD4+ cells could differentiate into two lineages and fates: IFN-γ and TNF producing Th1 cells, which were responsible for bacterial host defense, inflammation and autoimmunity and IL-4 producing Th2 cells, which were responsible for parasites defense and allergy (reviewed by Mosmann TR and Coffman RL 1989). Autoimmune diseases were classified according to which side the imbalance would be shifted: towards type-1 for diseases such as RA, IBD and also MS, towards type-2 for allergic diseases. To shift a committed immune system from type-1 to a type-2 immune response was the objective of many treatment trials for example in EAE. To support this concept, adoptive transfer of CD4+ cells that produce IFN-γ from a mouse with EAE to the same mouse strain causes EAE. In contrast, in models where IFN-γ is absent (knock-out for IFN-γ or IFN-γ receptor antibodies) EAE is more severe, in the absence of exaggerated Th2 responses. Also, both IL-12/IL-23-p40$^{-/-}$ and IL-23-p19$^{-/-}$ mice had abrogated EAE while IL-12-p35 subunit$^{-/-}$ mice had worse EAE (Becher B *et al* 2002, Cua D *et al* 2003), which showed that IL-23 and not IL-12 were responsible for CNS inflammation.

Recent evidence challenges the initial type-1/type-2 immunity interpretation and describes the differentiation of CD4+ T cell lineages from peripheral naïve CD4+ T cell precursor into three subsets of effector T cells, Th1, Th2 and Th17 (Veldhoen M *et al* 2006). IL-12 signaling through STAT4 allows lineage commitment to Th1 phenotype and production of IFN-γ (Szabo SJ *et al* 2000, Szabo SJ *et al* 2002). IFN-γ mediated STAT1 signaling leads to the induction of transcription factor Tbet and differentiation of Th1 cells. IFN-γ production is further potentiated by inflammatory cytokines such as IL6. IL-12 induced Th1 differentiation is more important to systemic and intracellular pathogens defense and delayed-type hypersensitivity responses. IL-4 mediated STAT6 signaling leads to the activation of transcription factor GATA3 and differentiation of Th2 cells to produce IL-13, IL-4 and IL-5 (Nelms K *et al* 1999). Th2 cells are important for production of immunoglobulin E and eosinophilic inflammation. The Th17 subset develops in response to IL-6 and TGF-β (Veldhoen M *et al* 2006), which signal through STAT3 and TGF-β receptor respectively to activate lineage-determining transcription factor RORγt. Th17

cells produce mainly IL-17A and IL-17F, in addition to IL-6, GM-CSF and TNF. IL-23 acts on memory $CD4^+$ cells to induce proliferation and maintain Th17 effector function and recruitment of Th17 cells to sites of inflammation, but it is not needed for differentiation. IL-23R is expressed only on memory and activated, not naïve T cells. The development of Th17 cells depends on co-stimulation with ICOS and maybe CD28 and is inhibited by IFN-γ and IL-4. IL-27 negatively regulates Th17 development by inducing Tbet. An even more recent study shows that IL-23 stimulation induces IL-21 and IL-22 secretion in Th17 cells. IL-21 is produced by activated T cells, including Th17 cells, and boosts IL-17 production. IL-21 also suppresses FoxP3 expression and Treg differentiation (Korn T *et al* 2007, Nurieva R *et al* 2007).

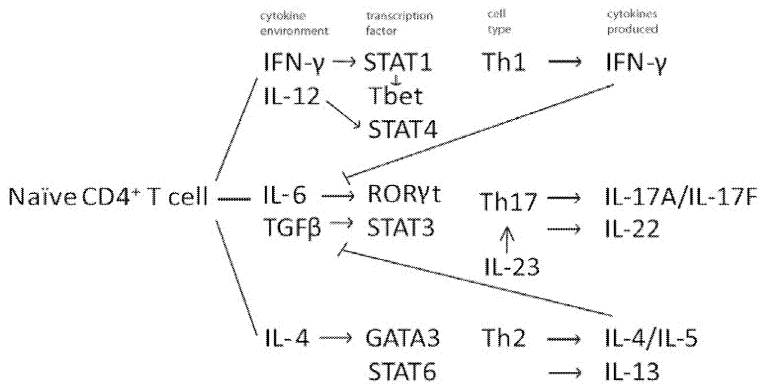


Figure 4. Differentiation of naïve $CD4^+$ T cells into helper T cells. In the presence of IL-12 there is activation of STAT4 which, together with the expression of Tbet induced by the activation of STAT1 by IFN-γ, causes T cells to adopt a Th1 phenotype and secrete more IFN-γ. The presence of IL-18 in the environment reinforces the IFN-γ production. In the presence of IL-4, GATA3 and STAT6 are activated and T cells adopt a Th2 phenotype characterized by increased secretion of IL-4, IL-5 and IL-13. In the presence of IL-6, which induces expression of the transcription factor RORγt, and TGF-β, which activates STAT3, cells adopt a Th17 phenotype and secrete IL-17A/F. IL-21, secreted by activated T cells and others, can also cause the differentiation into Th17 phenotype together with IL-6. IL-23 determines survival and causes expansion of Th17 cells.

The recent findings have moved the immunology paradigm towards a Th17 induced autoimmunity, with the role of IL-17 and IL-23 emphasized in most autoimmune diseases. Nevertheless, the features that previously made us think that EAE was a type1 immunity disease remain. Th1 cytokines are present in the brain lesions of acute EAE and mice that lack Tbet or STAT4 do not produce IFN-γ and are resistant to EAE. Crucially, IL-17^{+}IFNγ^{+}cells infiltrate the CNS during EAE (Korn T *et al* 2007). In humans, there are Th17 producing cells, but a specific lineage has not been identified. However, IL-17 has been implicated in MS (Matusevicius D *et al* 1999) and, together with Th17 cells, is an important new rediscovery.

Although they are fundamental to the balance and regulation of the immune system, regulatory cells will not be discussed in this thesis. Very briefly, many types of cells have immunoregulatory properties: IL-10 secreting Tr1 cells, TGF-β -secreting Th3, CD8+CD28-, CD8^{+}CD122^{+}, γδ-TCR T cells, NK cells, apoptotic neutrophils, coinciding with naturally occurring CD4^{+}CD25^{+}Treg. TGF-β induces FoxP3 expression in naïve CD4^{+} and their differentiation into induced Treg.

The new concepts challenged the previous paradigm and enabled a better understanding of some of the immune mechanisms in EAE. More remains to be found, as even these propositions cannot explain the variability in all congenic strains and individuals.

1.9 PATHOGENIC MECHANISMS SHARED WITH OTHER COMPLEX INFLAMMATORY DISEASES

Complex inflammatory diseases are a group of systemic or organ specific conditions that have a complex genetic etiology and an inflammatory pathology. The inflammatory systemic diseases include rheumatoid arthritis (RA), sarcoidosis, vasculitis (panarteritis nodosa, Churg-Strauss, …), atherosclerosis and system lupus erythematosous (SLE), the later being the only where autoantibodies (against self DNA, RNA or self-IgG) have a pathogenic role. The organ-specific diseases include diabetes mellitus type 1 (T1DM), inflammatory bowel disease (IBD - Crohn's and ulcerative colitis), asthma, thryroiditis (Graves' and Hashimotos's) and myasthenia gravis (MG). In MG and Graves', the autoantibodies are identified and correlated with

disease. Since the scientific community has used enormous resources in humans and models to study these other diseases, it is worth evaluating shared pathogenic mechanisms with MS.

Given the possibility that genes and mechanisms responsible for distinct inflammatory diseases can be shared, family members of people that have one such disease should have a higher chance of being affected by the same or any other inflammatory disease. This was evidenced in relatives of people with DM (Smyth DJ *et al* 2007). The clustering of MS cases in families is statistically confirmed but the clustering of other inflammatory or immune-mediated diseases in the same families still is controversial. Thyroiditis, psoriasis, inflammatory bowel disease, and rheumatoid arthritis are more frequent in families at risk for MS (Barcellos LF *et al* 2007) and auto-immune diseases are more frequent in first degree relatives of patients with MS especially if there were more cases of MS (Broadley SA *et al* 2000), but these observations were not confirmed in a population-based study (Ramagopalan SV *et al* 2007).

In animal studies, most models for MS are autoimmune (an autoantigen is injected) while that is not the case for experimental arthritis (only in collagen-induced arthritis is the autoantigen know). There is considerable overlap of the genetic regions linked to different inflammatory diseases development (Andersson A and Holmdahl R 2005), which could mean the control by the same gene. Furthermore, there are homologous loci between mouse, rat and human diseases, and loci that have an accumulation of QTLs for inflammatory diseases (Xu C *et al* 2001, Bäckdahl L *et al* 2003). In our laboratory, we use a distinct model for degeneration and inflammation, ventral root avulsion (VRA), a model for peripheral inflammatory neuropathy (EAN) which showed co-localization of QTLs with EA and EAE (Dahlman I *et al* 2001) and share resources with a group that works with EA. Animal resources, such as congenics and the AIL, are tested in all the models and candidate genes are cross-tested in cohorts of MS, RA and atherosclerosis and respective control groups. VRA is a standardized nerve trauma model that demonstrated a potent genetic regulation of nerve lesion-induced nerve cell death, local T cell accumulation and expression of MHC class II on microglia in a series of inbred rat strains, crosses and congenics (Olsson T *et al* 2005). This model was central in the identification and clarification of MHCIITA that was then show to be influential in MS, RA and cardiovascular disease (Swanberg M *et al* 2005).

The identification of IL7R and MHCIITA as genes associated with MS reinforces the position of MS as an immune mediated disease, and builds on the long established association with HLA DR2.

Systematic fine-mapping and positional cloning in the rodent models of different autoimmune diseases will define which genes are involved in several forms of autoimmunity and which genes are disease-specific. Similarly, large cohorts of patients with different immune-mediated diseases can be tested for the same candidate genes to determine which are shared and which are disease-specific. Some genes identified in other systemic and organ-specific inflammatory diseases, such as CTLA-4 and PTPN22, are not associated with MS, which may underlie the uniqueness of an immune disease that occurs in mostly sterile and immune privileged location.

2 AIMS

The major aim of my studies was to identify genes that regulate EAE and in this way clarify pathogenic mechanism of potential importance to multiple sclerosis. This may lead to recognition of new therapeutic strategies.

In particular, I concentrated on fine-mapping previously identified regions on rat chromosome 4 and rat chromosome 15.

The knowledge on innate immunity of MS still is scant. In a hypothesis-driven approach, I studied the role of toll-like receptors and the adaptor molecule MyD88 in MOG-EAE.

3 METHODS

3.1 ANIMAL MODELS: RATS AND MICE

All breeding and experimental procedures have been approved by the North Stockholm animal ethical committee. The animals are kept according to the rules of FELASA. The rats in inbred strains have identical genomes due to inbreeding of brother-sister pairs for over 20 generations. The strains used are LEW (Lewis), DA (Dark Agouti), PVG (Piedbald-Viral-Glaxo), ACI (A X C9935 Irish) and BN (Brown Norway) and the same ones with substitution of the MHC complex. Congenic strains were developed from different combinations of these with selection for rats with the "introduced" fragment followed by backcrossing and final intercrossing. To distinguish alleles with different parental origin, the markers must be polymorphic: aproximately 60% of microsatellites differ between DA and PVG, whereas only 10% differ between DA and ACI (Whitehead Institute). For the engineered mice, backcrossing with the parental strains is also mandatory so that the variable phenotypic effects depend on the mutation alone and not on random interacting contaminating DNA in the background. The phenotypes of the rats and mice can be compared with their parental strains or with their littermates, who will have the same degree of contamination. The crosses, in particular the AIL have been described elsewhere in this thesis.

Studies in rats all use MOG_{1-125} in IFA for the EAE induction protocol, whereas for mice we used MOG_{1-125} in CFA and pertussis toxin.

MOG_{1-125} is produced in our laboratory according to a modified protocol published by Amor S *et al*. The original MOG construct was a kind donation from Professor Christopher Linington, after transfection into *E.coli*. In brief, MOG-*E. coli* is cultured in LB medium at 37^{o}C, followed by addition of IPTG (isopropyl beta-D-thiogalactoside) inducer. The recombinant protein forms inclusion bodies which are solubilized and the protein harvested, washed and resuspended in 8M urea solution. rMOG is then extraxted by chelate chromatography using Hitrap Chelating Sepharose Fast Flow stationary phase and 1% NiCl solution mobile phase. MOG suspension is then dialyzed to a

biological buffer (i.e. PBS or HEPES) and purified by ion exchange chromatography.

EAE signs and body weight are monitored daily from day 9 until day 30-40 post immunization (*p.i.*), when the animals are euthanized with CO_2. The scale for EAE scoring usually is: 0, healthy; 1, tail weakness or tail paralysis; 2, hind leg paresis or hemiparesis; 3, hind leg paresis or hemiparesis; 4, hemi or tetraplegy and 5, moribund. The following clinical parameters are assessed: incidence of EAE *i.e.*, signs of EAE present for more than one day; onset of EAE *i.e.*, day of first clinical sign; duration of EAE *i.e.*, number of days rats showed clinical signs; maximum EAE score, cumulative EAE score, *i.e.*, sum of the EAE scores obtained from day nine until the end of experiment.

3.2 INTERVAL MAPPING AND RQTL/STATISTICS

A QTL study initially involves selection of appropriate parental inbred strains, establishment of a backcross or F2 intercross, phenotyping and genotyping of progeny for polymorphic markers that are evenly spaced throughout the genome, and regression analysis or other linkage statistical methods. The goal is to identify genomic regions in which allelic variation is associated with phenotypic variation. For detecting modifiers of a specific disease phenotype, one of the parental strains should carry a disease-causing allele. Genotyping used microsatellite markers and their physical positions were obtained from public databases such as Ensembl or NCBI. PCR amplification with [γ-^{33}P]ATP end-labelling of the forward primer products were size fractionated in 6% polyacrylamide gels and visualized by autoradiography. All genotypes were evaluated manually. Lately we use fluorescently labeled microsatellite markers and avoid the radioactive genotyping.

For the AIL data, the multiple imputation method (Sen and Churchill 2001) implemented in the R/qtl statistical software (Broman K *et al* 2003) allowed us to perform interval mapping to identify the QTLs of main effect, two-dimensional scans with a two QTL model and fit multiple QTL model tests. R/qtl assumes that our data results from an F2, but with the imputation method and very dense genotyping the gives acceptable results. The two-dimensional scans with a two QTL model examine all pairs of markers and intermarker positions for association with a given phenotype, to identify additive effects and epistatic interactions between the loci. The fit multiple QTL modelling allows the

statistical validation of the independent effect of each identified QTL and its interactions. It does so by subtracting the effect of each QTL or QTL interaction and comparing that model to the initial model of phenotypic variance where all QTLs have a full effect. For determination of significance thresholds we computed the family means and residuals from the family mean for each rat and repeated the one- and two-dimensional scans to record the maximum LOD score determined by the family effects for each phenotype. The maximum LOD scores for the original one-dimensional scan were all higher than 3.4 (Lander and Kruglyak 1995). The conventional permutation method (Churchill and Doerge 1994) should not be used in the AIL due to the different family structure of the G10 compared to F2 generations. To determine confidence intervals we tried the bootstrapping method (Manichaikul *et al* 2006, Visscher PM *et al* 1996) which did not work in these studies due to the existence of several close QTLs. We also tried Bayesian HPD intervals implemented using R/qtl and R/qtlbim (Yandell BS *et al* 2007). The LPD is designed so that location of the QTL is the parameter of interest, and the HPD intervals would be more accurate to define confidence intervals (Sen and Churchill 2001). Again, the data showed that the QTLs defined in these studies are closely situated and linked, the probable the reason why there were only one interval for each phenotype and we were not able to draw conclusions from such analysis. Finally, we used 1-LOD support intervals, and defined the limits as the location of the closest genotyped marker outside the interval. Our choice was based on Lander and Botstein (Lander and Botstein 1989), but also on the comparisons between 95% Bayes credible intervals and 1-LOD support intervals (Manichaikul *et al* 2006).

3.3 PRIMARY CELL CULTURES FROM SPLEEN AND LYMPH NODES

Splenocytes and lymph node cells were cultured in DMEM supplemented with 10% Fetal Calf Sera, 100U/ml penicillin, 100µg/ml streptomycin, 292µg/ml L-glutamine (DMEM complete) (all from Invitrogen, Carlsbad, CA) with or without MOG_{1-125} and 20µg/ml polymyxin B sulphate (Sigma-Aldrich) for different amounts of time at 37ºC and 5% CO_2.

3.4 SORTING CELLS WITH SURFACE MARKERS (MACS, MOFLO, FACS) OR INTRACELLULAR STAINING (FACS)

MACS MicroBeads™ are coupled to highly specific antibodies or proteins. Cells are isolated by placing the MACS Column inside the magnetic field of the MACS Separator. The selection can be positive, only cells that attach to the antibody are retained in the column and are eluted when the magnetic field is removed, or negative, if the aim is to deplete the flow-through of the population that attached to the antibodies. The cells will be bound to the MicroBeads in the first case, but unlabelled in the second. The purity must be checked by FACS and can be enhanced with several rounds of sorting.

FACS – fluorescence activated cell sorting based upon the specific light scattering and fluorescent characteristics of each cell. To characterize or identify cells that have a particular surface marker, we stain them with unconjugated or biotin conjugated monoclonal and polyclonal antibodies or antibodies linked to fluorescent dyes.

MoFlo – high performance cell sorter flow cytometry - provides sorting precision required for the isolation of rare-cell populations with high purity and yield. The cells are stained in the same way as for FACS. Sorted cells are viable, purity is improved and cells can be sorted into distinct populations.

Intracellular staining –This is a good way to measure numbers of cells that produce certain cytokines. *In vitro* stimulation of cells is usually required for detection of cytokines by flow cytometry since their levels are typically too low in resting cells. Stimulation of cells with the appropriate reagent will depend on the cell type and the experimental conditions. According to the supplier's information, to stimulate T cells to produce IFN-γ, TNF, IL-2, and IL-4, a combination of PMA (a phorbol ester / PKC activator) and Ionomycin (a calcium ionophore) or anti-CD3 antibodies can be used. To induce IL-6, IL-10 or TNF production by monocytes, stimulation of PBMC's with lipopolysaccharide (LPS) can be used. Further, it is necessary to block secretion of cytokines with protein transport inhibitors, such as monensin or brefeldin A, during the last few hours of the stimulation in order to detect intracellular cytokines. Cells are first stained for surface antigens, then fixed with paraformaldehyde to stabilize the cell membrane and permeabilized with the detergent saponin to allow anti-cytokine antibodies to stain intracellularly. The FACS analysis is similar to the previous techniques. In Study IV, to measure IL-17 producing cells, splenocytes were

instead stimulated with a combination of 500ng/ml phorbol dibutyrate (Sigma-Aldrich) and 500ng/ml ionomycin (Sigma-Aldrich) and blocked with brefeldin A (BD Biosciences) for 4h at 37ºC, as the stimulation with PMA had killed most of them in a previous experiment. Cells were fixed, permeabilized and incubated with rat anti-mouse CD16/CD32 (BD Pharmingen) to prevent unspecific binding of antibodies to Fc receptors. The following antibodies were used for staining: anti-CD3-FITC, anti-CD4-APC; anti-IL17-PE, anti-IFNγ-PE or rat isotype control IgG1-PE (all from BD Biosciences). Cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences) using Cellquest software (BD Biosciences).

3.5 REAL TIME QUANTITATIVE -PCR

RT-PCR amplifies a defined piece of a RNA molecule. The RNA strand is first reverse transcribed into its complementary DNA (cDNA), followed by amplification of the resulting DNA using polymerase chain reaction. In the “first strand reaction”, cDNA is made from mRNA template using dNTPs and a RNA-dependent DNA polymerase or reverse transcriptase. “Second strand reaction”, or standard PCR, is initiated: the cDNA, DNA polymerase and DNA primers are heated above 95ºC to separate the cDNA strands and cooled to around 60 ºC to allow specific sequence binding of the primers. Further heating allow the DNA polymerase to make double stranded DNA, until the step at 95ºC separates the two DNA strands. And the cycles repeat.
Real-time PCR, also called RT-PCR or quantitative PCR, simultaneously amplifies and quantifies a DNA molecule. The amount of DNA is quantified at each round either by modified DNA oligonucleotide probes that fluoresce when hybridized with a cDNA (TaqMan® probes, Applied Biosystems, are the ones used in our lab) or by fluorescent dyes that intercalate with double-stranded DNA (SYBR® Green, Molecular Probes). TaqMan probes depend on the 5'-nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon because they are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moeity coupled to the 3' end. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, the polymerase cleaves the probe from its specific binding to cDNA, which decouples the fluorescent and quenching dyes. Fluorescence increases in each cycle, proportional to the

amount of probe cleavage. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA and upon excitation emits light. SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products. Similar to the primers for TaqMan PCR, the primers pairs in “SYBR-PCR” must be designed to be specific, preferably over an exon border and not create primer-dimers, but the PCR products should still be run in a gel to insure one band only and sequenced. The other methods to detect mRNA, Northern blot and RNase protection assays, are more time-consuming and do not allow precise quantitation. In order to quantify each sample, the results can be compared to a standard curve or with the comparative Ct method. The standard curve contains sequential dilution from a known amount of the target cDNA or just a sample that contains the target in approximately the same amount and media as the unknowns to mimic the efficiency of the transcription step and yields information on relative changes in the expression. To relate the amount detected with the amount of cDNA in the original sample, we normalize with a housekeeping gene. The choice of housekeeping gene dependes on the cells and species, but also if the cells were cultured and differentially stimulated: the amount of housekeeping gene should not change and relate only to the amount of RNA of the cells in the first place. The comparative C_t method involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene.

3.6 ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY) IN SERA AND SUPERNATANTS

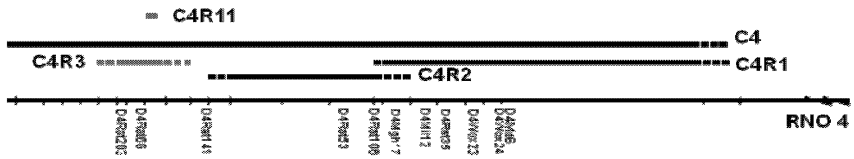
ELISA is used to detect the presence of an antibody or an antigen in a sample. **Sandwich *ELISA***. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody specifically; (3) detection antibody is added, and binds to antigen; (4) if the detection antibody is not conjugated with biotin, for example, an enzyme-linked secondary antibody is added, which binds to detection antibody; (5) substrate, ABTT or TMB, is added, and is converted by enzyme to a detectable form either

chromogenic or fluorogenic; (6) quantification of the result using a spectrophotometer or spectrofluorometer.

MOG ELISA (or any other antibody in sera). (1) Plate is coated with MOG against which the immune response was mounted; (2) sera samples are added, and any antibodies present bind to antigen specifically; (3) detection antibody is added, and binds to antibodies; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is converted by enzyme to a detectable form.

4 RESULTS

Our strategy was to fine map and identify, in an unbiased way, genes or small genetic regions which may be tested further for their influence in MS. We focused on the C4R3 and C4R11 regions depicted below on rat chromosome 4 (RN04), which are known regulate neuroinflamation and are rich in immune-related genes.



4.1 *EAE20-EAE22*, ON THE TELOMERIC REGION OF RN04, INFLUENCE MOG-EAE, AS EVIDENCED BY THE ANALYSIS OF MOG-EAE IN G10 AIL AND CONGENICS.

In Paper 1, high-resolution mapping of the above C4R3 region in G10 (DA x PVG.1AV1) AIL identified *Eae20* and *Eae21*, two closely situated loci. *Eae20* is linked to incidence and onset of EAE, maximum EAE score and weight loss. *Eae21* is linked to duration of EAE, maximum and cumulative EAE score and weight loss.

Furthermore, evidence for QTL interactions was drawn from two-QTL model analysis over the 16.8 Mb region. It achieved the identification of *Eae22*, a MOG-EAE QTL where the effect of the genes had not been enough to be detected on its own, but only when acting in concert with *Eae20*. The effects of the QTLs and the epistatic interaction were confirmed with a model of phenotypic variance that initially included all fractions but would drop the effect of one at a time and compare with the initial model. The independent effect of *Eae20* was confirmed in all phenotypes as was the effect of the *Eae20*:*Eae22* interaction. The effect of *Eae21* was confirmed in all phenotypes but incidence

and onset of EAE. The effect plot of the interaction between *Eae20* and *Eae22* shows that the combination of homozygous alleles from the resistant PVG strain at *Eae20* and susceptible DA alleles at *Eae22* has a more severe effect on cumulative EAE score, and other phenotypes, than DA alleles in both loci. This combination occurs in congenic strain tested by Becanovic *et al* (DA-PVG-*chromosome 4R3*), where it showed worse EAE, but also in the smaller congenic strain tested here. The MOG-EAE experiment on a congenic strain that contained PVG alleles spanning 1.44 Mb (DA.PVG-*chrom4R11*) in a DA background conferred protection from those genes on susceptibility and severity of EAE. Our interpretation of these apparently conflicting results proposed two possibilities: *Eae20* and *Eae21* have an interaction that was not identified where DA alleles or PVG alleles at *Eae21* are respectively protective or deleterious or there is more than one EAE gene within *Eae20* and the one that has DA alleles in DA.PVG-*chrom4R11* interacts to protect from EAE.

In addition to the results in Paper 1, some experiments were done and not published. Most of the genes in the region transferred from PVG into DA. PVG-*C4R11* rats are expressed in antigen presenting cells (APC). The antigen uptake and processing by APCs could be impaired in the congenic rats. We compared the DA.PVG-*chrom4R11* and parental DA rats immunized with the immunodominant MOG peptide 91-108 in CFA (Weissert R *et al* 2001) and found that the congenic strain also had less severe EAE (Graph and Table below), which did not support the hypothesis.

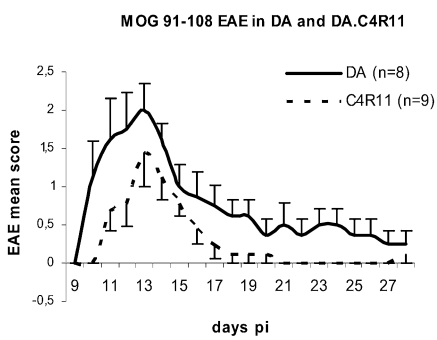


Table. Incidence and severity of MOG $_{91\text{-}108}$-induced EAE in DA and DA. PVG-*C4R11* rats

Strain	Incidence	Maximum score	Cumulative score	Mortality
DA	8/8 (100%)	2,25 (±0,3)	15,4 (±3,5)	0/8
DA. PVG-*C4R11*	7/9 (78%)	1,67 (±0,4) ^{ns}	5,9 (±1,5) *	0/9

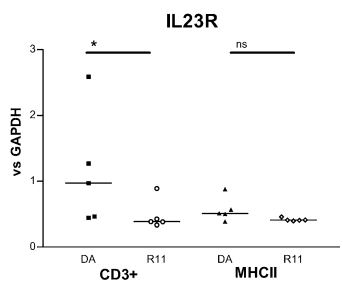
a) Mann-Whitney U test was used to calculate p values for maximum score and cumulative score. Significance scores are according to ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$

b) Maximum and cumulative EAE scores were calculated for both affected and unaffected rats and presented as group mean with standard error

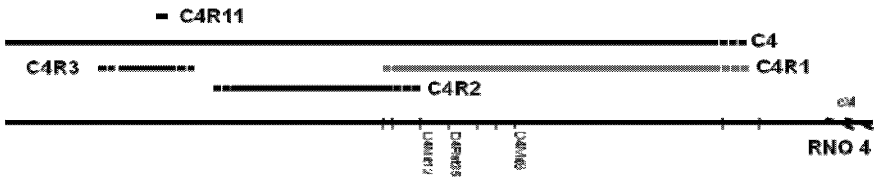
c) Sum of each daily score for the 27 days

To study potential mechanisms explaining different EAE results, we used $MOG_{1\text{-}125}$ immunized lymph node cells (LNC) at day 10 p.i. to measure expression of mRNA of inflammatory cytokines. We checked both in LNC ex-vivo and after MACS sorting of MHC class II $(OX6)^+$ and $CD3^+$ cells. At this time point, there were no signs of a type 1 or type 2 bias in LNC, as neither IFNγ nor IL-10 were differentially expressed in DA.PVG-*C4R11* compared to DA rats. IFNγ-inducible-protein, CXCL10 or IP-10, is also involved in promoting Th1 responses and was not differentially expressed in these experiments. IL-1β expression was significantly lower and there was a tendency towards lower IL-6 expression in $OX6^+$ from DA.PVG-*C4R11* rats. The receptor for IL-12 and IL-23 are heterodimers composed of a common IL-12Rβ1 subunit and either an IL-12Rβ2 (for IL-12receptor) or an IL-23R subunit (Presky DH 1996, Parham C 2002), respectively. These receptors are expressed on T and NK cells and also on APCs (Li J *et al* 2003). The expression of IL-23R was strongly reduced in T cells from DA.PVG-*chromosome4R11* compared to DA rats.

IL-23 is responsible for survival and expansion of Th17 cells in autoimmune diseases. Hypothetically, a gene within DA.PVG-*chromosome4R11* controls a pathway that results in a low expression of IL-23R on T cells and this may explain the protection from EAE in the congenic strain.



4.2 *EAE24-EAE28*, LOCATED ON THE CENTROMERIC REGION OF RN04, REGULATE MOG-EAE.



MOG-EAE effect in congenic substrains

We established two recombinant congenic strains derived from DA.PVG-*C4R1*, *i.e*. DA.PVG-*chromosome 4R4* and DA.PVG-*chromosome 4R5* (**within C4R1 in the Figure above**). These two strains were subjected to MOG-EAE, along with the parental DA strain. The DA.PVG-*chromosome 4R5* rats did not show any significant differences in EAE phenotypes when compared to DA. This was surprising as they contain a larger PVG insertion than DA.PVG-*chromosome 4R4* rats, which displayed less severe EAE with a lower cumulative score, a tendency towards lower maximum EAE score and a later onset of EAE than the DA rats (Table below). The experiment was repeated twice and the effect in DA.PVG-*chromosome 4R4* was confirmed, although differences in sample size and MOG-EAE induction protocol may have contributed to discrepancies in the statistical significance between the different experiments. Together, our data suggest that several genes in the 58 Mb region and possible epistatic interactions regulate EAE. To clarify such interactions and to further improve mapping resolution, we dissected the C4R1 region in the AIL.

The full length DA.PVG-C4R1 rat congenic strain was intercrossed with parental DA rats to create heterozygotes that were bred to obtain recombinant strains with smaller PVG fragments. The following recombinant strains were tested in MOG-EAE (Figure 1): DA.PVG- *chromosome 4R4* (homozygous PVG for *D4Rat23-AMP* on a DA background) and DA.PVG- *chromosome 4R5* (homozygous PVG for *D4Rat23-D4Rat38* on a DA background) and DA. In each experiment, between 10 and 28 female rats were used per strain, and they were immunized with MOG_{1-125} from different batches.

Table. Summary of the clinical EAE outcome in recombinant congenic strains vs DA

Experiment and strains	Incidence	Mean day of EAE onset ± sd	Maximum EAE score ± sd	Mean cumulative EAE score ± sd
I.				
DA	23/25	14.3±5.1	2.87±1.1	47.1±32.6
DA.PVG-*R4*	22/28	16.3±6.6**	2.46±1.49	22.3±26.7***
DA.PVG-*R5*	21/25	13.0±6.5	2.70±1.5	41.3±37.1
II.				
DA	11/18	24.5±13.1	1.64±1.5	20.9±24.8
DA.PVG-*R4*	3/10	32.8±11.6	0.6±1.0	3.15±5.7*
DA.PVG-*R5*	6/19	31.7±2.9	0.89±1.5	10.58±18.3
III.				
DA	17/18	16.7±6.3	3.14±1.0	35.5±14.5
DA.PVG-*R4*	9/10	18.2±7.9	2.63±1.1	26.6±15.1

*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ sd = standard deviation

The same region had been analyzed with linkage analysis in the data from the MOG-EAE experiment in G7 (DA x PVG.1AV1) AIL (Paper 3). Briefly, 1068 G7 rats that were immunized with MOG of which genotyping was done on 152 rats that were sick with EAE and 162 random healthy rats. The recombination events in the genotyped rats were enough to define two QTLs with significant LOD score for all the clinical phenotypes and a QTL linked to anti-MOG IgG2b in the telomeric part of the region. The fact that the results from the MOG-EAE experiments in the congenic substrains were not clear and the availability of the data on the G7 (DA x PVG.1AV1) AIL made us continue the study (Paper 2).

In Paper 2, high resolution mapping of the 58 Mb C4R1 region in G10 (DA x PVG.1AV1) AIL identified *Eae24-Eae28,* five distinct EAE-regulating QTLs. DA alleles at the peak marker locations were generally disease promoting and, while females were more affected than males, there were no sex specific QTLs in the region. Distinct sets of QTLs within *Eae24-Eae28* co-segregate with susceptibility or severity phenotypes. *Eae24*, *Eae26* and *Eae28* were linked to incidence and onset of EAE. *Eae24*, *Eae25*, *Eae26* and *Eae27* were linked to maximum and cumulative EAE score and duration of EAE. *Eae25* influences mostly severity and *Eae28* influences mostly susceptibility. Unlike in the previous study, no new QTLs were identified by the two-QTL analysis in the region. The interaction between *Eae24* and *Eae26* influenced susceptibility phenotypes. This was confirmed by the comparison between an

initial model of phenotypic variance which included the individual effects from the three independent QTLs and the interaction and models that excluded each component. The analyses of epistatic interactions for the severity phenotypes were more complicated because the model with the best result showed interaction of three QTLs, *Eae24*, *Eae25* and *Eae26*. These QTLs are closely positioned and linked, and most analyses cannot evaluate them.

Anti-MOG antibody levels for IgG1 are linked to *Eae24*. This is the first time that this immunological subphenotype shows significant linkage to a locus that also shows an effect from clinical EAE phenotypes.

4.3 *EAE19,* ON RAT CHROMOSOME 15, REGULATES MOG-EAE.

The locus on rat chromosome 15 that showed suggestive linkage to SC in CFA-induced EAE was also identified in MOG-induced EAE and experimental autoimmune neuritis (EAN) by Dahlman *et al*. A 25 cM segment of ACI alleles was transferred into a susceptible DA background, creating the full length DA.ACI-*chromosome15* (C15) congenic strain. Subsequently, several other sub-strains containing smaller segments within the same region were created and all were tested for MOG-EAE. ACI alleles in the DA.ACI-*chromosome15*, in the DA.ACI-*chromosome15R3b* and DA.ACI-*chromosome15R4* congenic strains confer a degree of protection to MOG-EAE, while the ACI alleles in DA.ACI-*chromosome15R1* and DA.ACI-*chromosome15R3a* could not make a difference compared to the DA strain. This narrowed the region that regulates MOG-EAE to a segment smaller than 2.5 Mb (from D15Rat23 to D15Rat71). Because the MOG-EAE experiments were not done simultaneously on all sub-strains, we normalized the results of the six experiments. The data shown in the graphs (Figure 2 in Paper 3) refer to the mean residual values for each group of rats after the mean maximum and mean cumulative value for the sub-strain experiments.

Linkage analysis in the G7 (DA x PVG.1AV1) AIL MOG-EAE experiment resolved the DA.ACI-*chromosome15* (C15) 53 Mb long region into a significant locus, *Eae19* that is characterized by linkage to both severity and susceptibility phenotypes. The 1-LOD support confidence interval is 13 Mb long, which then contained 32 and now contains 48 confirmed or predicted genes.

Eae19 is thus characterized by the linkage analysis data for peak marker and confidence interval. The effect from the ACI alleles contained in the DA.ACI-*chromosome15R4* interval that overlap the centromeric 2.8 Mb of the confidence interval up to D15Rat71 was confirmed in the congenics experiment.

4.4 REGULATORY ROLES OF TLR4 AND TLR9 IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

To investigate how upstream TLR signals influence autoimmune T cell responses, we studied the role of individual TLR and MyD88, the common TLR adaptor molecule, in the initiation of innate and adaptive immune responses in EAE. WT C57BL/6, TLR4-, TLR6-, TLR9- and MyD88-deficient mice were tested in several experiments that are shown in the Table below. Proinflammatory cytokine expression was measured in purified mDC, pDC, T cells and sera from individual mice to link upstream TLR-based signals to disease-inducing mechanisms.

Table. Clinical signs of EAE induced by MOG/CFA in C57BL/6, MyD88$^{-/-}$ and TLR$^{-/-}$ mice

Experiment	Mouse strain	Incidence of EAE	Mean day EAE onset	Mean accumulated EAE score[A]	Mean peak EAE severity[B]
1	C57BL/6	4/8 (50%)	16.0 +/- 0	14 +/- 7	1.3 +/- 0.6
	MyD88$^{-/-}$	0/5 (0%)	N/A	0	0
	TLR9$^{-/-}$	5/6 (83%)	16.6 +/- 0.2	24 +/- 9	2.2 +/- 0.5
2[C]	C57BL/6	7/7 (100%)	13.0 +/- 0	ND[D]	3.1 +/- 0.1
	MyD88$^{-/-}$	0/7 (0%)	N/A	-	0
3	C57BL/6	7/7 (100%)	15.5 +/- 0.2	14 +/- 4	1.4 +/- 0.5
	TLR9$^{-/-}$	6/7 (86%)	16.2 +/- 1.0	21 +/- 6	1.6 +/- 0.5
	TLR6$^{-/-}$	6/7 (86%)	15.0 +/- 0.7	18 +/- 6	1.4 +/- 0.5
4	C57BL/6	4/7 (57%)	17.0 +/- 0.7	9 +/- 4	1.1 +/- 0.5
	TLR4$^{-/-}$	6/7 (86%)	17.0 +/- 0.5	19 +/- 5	2.1 +/- 0.6
5	C57BL/6	12/12 (100%)		25 +/- 2.5	2.3 +/- 0.7
	TLR4$^{-/-}$	8/8 (100%)		35 +/- 0.7	3.4 +/- 0.3
	TLR9$^{-/-}$	14/14 (100%)		36 +/-1.3	3.4 +/- 0.2

Mice were immunized with 200 µg MOG_{1-125} in CFA followed by pertussis toxin injections on d 0 and 2. Mice were monitored for clinical signs of EAE until d 32 after immunization.

[A]Mean accumulated EAE score is the mean cumulative score +/-SEM on d 32 after immunization for both affected and unaffected mice. [B]Mean maximum EAE score +/-SEM for both affected and unaffected mice. [C]Mice were immunized with 250 µg MOG in CFA to induce a more severe EAE disease. [D]Mice were sacrificed on d 13, because the mice developed acute disease.

MyD88$^{-/-}$ mice were totally resistant to immunization with MOG_{1-125} in CFA and pertussis toxin while C57/BL6 (WT) mice developed chronic signs of EAE. Thus, induction of EAE requires MyD88-dependent signaling pathways.

Proinflammatory cytokine expression was evaluated in CD11c^{hi}B220^{-}Gr-1^{-} mDC and CD11c^{lo}B220^{+}Gr-1^{+} pDC from spleens after MOG immunization. MyD88 was shown to mediate IL-6 and IL-23/p40 subunit but not IL-23/p19 subunit expression in mDC. In comparison to WT mice, T cells (Thy1.2^{+} MACS sorted), *ex-vivo* from MyD88$^{-/-}$ mice fail to express IL-17 and the frequency of CD3^{+}IL17^{+} (FACS) cells was reduced. Accordingly, MyD88$^{-/-}$ splenocytes stimulated with MOG for 48 hours did not express IL-17. IL-23 receptor mRNA expression in Thy1.2^{+}cells was much lower in MyD88$^{-/-}$ than in WT mice, but TGF-β, which is said to be the inducing factor, had similar levels. The protein levels for IL-6 and IL-17 in sera were evaluated with ELISA. The early IL-6 responses are MyD88 independent and the day 10 pi are MyD88 dependent, as shown by the up-regulation at day 2 pi in both MyD88$^{-/-}$ and WT mice and only in WT mice at day 10. Serum IL-17 is only up-regulated at day 10 in WT mice and absent or much lower in MyD88$^{-/-}$ mice.

MOG-EAE was consistently exacerbated in mice that lacked TLR4 compared to WT mice. The splenic mRNA expression of IL-6, IL-23p40 and IL-23p19 was several-fold higher in mDC from TLR4$^{-/-}$ than WT mice, which indicates that TLR4 signals regulate IL-6 and IL-23 production by mDC during initiation of EAE. By adding *Mycobacterium tuberculosis* (MT) to mDC from WT and TLR4$^{-/-}$ mice we showed that MT was not directly responsible for the IL-6 and IL-23 induction. A sign that a worse EAE response was being mounted was that the TLR4$^{-/-}$ mice had a higher frequency of CD3^{+}IL17^{+} cells in the spleen at day 10 pi and the mRNA expression of IL-17 was twice as high in Thy1.2^{+} splenocytes, compared to WT. At day 10 pi, serum IL-17 levels were also higher in TLR4$^{-/-}$ compared to WT mice. Concordant with the fact that a Th1 response was down-regulated while the Th17 response was being mounted, the mRNA expression of IFN-γ is lower in Thy1.2^{+} splenocytes from TLR4$^{-/-}$ compared to WT mice.

TLR9$^{-/-}$ mice developed more severe MOG-EAE than WT mice showing that TLR9, like TLR4, regulates rather than promotes MOG-EAE. Serum IL-17 levels were higher in TLR9$^{-/-}$ than in WT mice, but we could not detect a differential expression of mRNA from Thy1.2^{+} splenocytes or splenocytes

cultures with MOG for 48 hours or a higher frequency of $CD3^{+}IL17^{+}$ splenocytes, which argues for other cells being also responsible for its production. IL-6, IL-23 and IFN-γ were unaffected in DC, but IL-6 production by splenocytes after 48 hours MOG stimulation and in the serum at day 10 was upregulated in $TLR9^{-/-}$ compared to WT mice.

5 DISCUSSION AND FUTURE PERSPECTIVES

STUDIES IN AIL AND EPISTATIC INTERACTIONS

High resolution mapping of regions on rat chromosome 4 (RN04) and rat chromosome 15 (RN15) with data from the MOG-EAE experiments in the 7th and 10th generation of the AIL between susceptible DA and resistant and MHC similar PVG.AV1 confirmed the influence of these regions on MOG-EAE. In both chromosomes the confidence intervals for the QTLs were significantly reduced. In gene sparse regions, the number of recombinations in the 7th generation (G7) of the AIL was sufficient to reduce the number of candidate genes for further studies. In gene dense regions, such as RN04, the extra recombinations obtained with the 10th generation (G10) are needed for a better characterization of the QTLs. We continued the AIL breeding into further generations but performed no more MOG-EAE experiments. The fact that some regions became “fixed” and lost Hardy-Weinberg equilibrium despite the random mating could cause spurious results.

Most regions studied show linkage both in the G7 and G10 MOG-EAE experiments. This congruity may not always happen because the experiments had some differences. For example, only 10% of rats had signs of EAE among the 1068 in the G7 experiment whereas one third of 794 had signs of EAE in the G10. Furthermore, all the G10 rats were genotyped whereas, in the G7, only the sick rats and a control healthy group in a total of 314 were selected.

Both regions on RN04 turned out to be composed of more than a single QTL. It is possible that the original linkage was detected because of the effect from more than one gene. These genes may co-localize randomly or be part of the same regulatory pathways. As they may belong to the same pathway, possible interactions between the genes were investigated. In Paper 1 and Paper 2 the investigation of epistatic interactions showed that, in addition to the independent effects of each QTL, the interaction between QTLs had a major effect on most EAE phenotypes. In Paper 1, *Eae22* did not have an independent effect but was identified when the epistatic interactions were considered. In Paper 2, all the locations involved in epistatic interactions were

also detected as independent QTLs. A possible explanation is that the region investigated has many closely located independent QTLs and almost no area outside the region was genotyped and analyzed. Also, the number of recombinations in G10 may still be too low for some epistatic interactions to be detected, if they happen to occur between very closely located genes. Further, the algorithms in most statistical programmes have trouble to handle closely situated linked genotypes or locations.

The interaction analysis revealed very interesting features of the genomic structure. Not only was *Eae22* identified (Paper 1), but also distinct sets of QTLs interacted to regulate distinct phenotypes (Paper 2). The concept that most genes and gene products work in networks is widely accepted. Further, some of the networks or pathways are regulatory and the net effect does not reflect the sum of the effects of the individual components. A deviation from the additive effect can be considered as epistasis. This is the case with most biological phenotypes, but to test the hypothesis we must build models that represent these complex interactions. So far, in our laboratory, this has been done only for small genome regions of interest.

The RN04 DA.PVG-*C4R1* region (Paper 2) was also investigated in other experimental models such as experimental autoimmune neuritis (EAN) and one model for experimental arthritis, but no clinical phenotypes showed linkage (unpublished results).

Other possibilities to further pursue the AIL strategy include the use of different strain combinations, the careful record and evaluation of family structure over the generations and a more complete characterization of quantitative phenotypes. These experimental designs help to increase the statistical power together with the appropriate number of rats in experiment and full and careful genotyping that were dealt with in the AIL G10. To increase the data extracted from experiments in crosses, measurement of gene expression levels with microarrays in new experiments enables eQTL mapping. The interactions mentioned in this thesis are evaluated over small chromosomal regions. But the interactions between different chromosomal regions already genotyped deserve further assessment to find if they are working at longer distance. The results of the interaction analyses at a genome wide level will be much more valid but only feasible in F2 crosses. In addition, some environmental influences can be included in the models. Overall, studies of

genomic regions without a biased choice of genes are critical for identification of new pathogenic pathways but may also reveal intergenic regulatory regions of importance that would otherwise be missed.

MOG-EAE IN CONGENIC STRAINS

Congenic strains were used to narrow and confirm the effect of QTLs. To confirm the QTL effect, our congenic strains isolated resistant alleles from the QTL confidence interval in a susceptible background, assuming they cause the congenics to be less susceptible than the susceptible parental strain. This protection is easier to detect, but the opposite combination of alleles can be tested in reciprocal strains. The validity of the differences between congenic and parental strains for the effect of the transferred genes assumes a homogeneous background after many backcrosses. In Paper 1, the number of PVG genes in DA.PVG-*chrom4R11 rats* was much smaller than in the *Eae20* confidence interval, but the effect of *Eae20* was confirmed. The subsequent characterization of the protection in immune mechanisms of the DA.PVG-*chrom4R11* is informative about the pathways involved. The objective to achieve single gene congenic strains is hindered by regions of low recombination rates as well as region where the alleles from the two parental strains are similar thus cannot be distinguished. Despite the arguments on possible interactions, some authors still believe congenic strains are the golden standard for the definition of a QTL, whereas linkage analysis results may be spurious.

In Paper 3, congenic strains that overlap at different locations are compared to each other and to parental strain after induction of MOG-EAE. The segment of donor alleles shared by the strains that are consistently less susceptible to MOG-EAE than the parental strain is considered to be regulating disease. The common segment is included in the 1-LOD support confidence interval for *Eae19* defined by the linkage analysis in the G7 AIL thus confirming the effect of the QTL. The strategy to compare overlapping congenic strains is referred to as “congenic mapping” and can be used to define QTLs. This is only possible when the region harbors genes that have independent effects but do not have epistatic interactions. For example, the congenic strains tested for the region studied in Paper 1 have paradoxical effects. The strain with more “protective” alleles has more severe EAE while the strain with less of the same

“protective” alleles is protected, in comparison to the parental strain. Furthermore, the overlapping congenic strains tested for the region studied in Paper 3, did not have consistent protection, despite including alleles that are shown to regulate MOG-EAE by linkage analysis. In both cases the search for epistatic interactions revealed evidence for the independent effect from the interactions themselves. In regions that have genes with epistatic interactions the comparisons between overlapping strains must be interpreted with caution.

Congenic strains are nevertheless an invaluable tool to learn about the mechanisms and functions that may be involved in disease. For *Eae20* and *Eae26*, congenic sub-strain containing fewer PVG alleles are being characterized and this study would further benefit by inclusion of gene expression microarray data of inbred and congenic strains.

The genes contained in the confidence intervals of the MOG-EAE QTLs that are part of the results of this thesis, *Eae19-Eae22* and *Eae24-Eae28*, are not listed and are not described in detail. The lists would be too long and any discussion at this point would be biased. The genes can be retrieved from public databases such as Ensembl.org and NCBI and the species comparisons can also be made on-line.

INNATE IMMUNITY IN MOG-EAE

Our study on the role of innate immunity at the initiation of MOG-EAE adaptive immune response shows the requirement for MyD88 signaling in this MS model. This may support the importance of a microbial component in the etiology of MS. The regulatory roles shown by TLR4 and TLR9 are novel and these data conflict with a previously published study in MOG peptide EAE where the absence of these receptors abrogated EAE. The characterization of immune molecules in sorted mDC and T cells confirmed the more severe EAE phenotype in TLR4$^{-/-}$ mice compared to WT, but was not as clear in TLR9$^{-/-}$ mice. TLR9 are expressed in the endosomal not cellular membranes and the immune mechanisms may be dependent on yet unknown pathways that regulate neuroinflammation.

The immune characterization of these rodents reflects new concepts in the immunopathology of EAE, as mentioned in the Introduction. With respect to these concepts, we need to reevaluate the immune mechanisms of EAE in

many rat congenic strains studied in the past. One concern is the use of *Mycobaterium tuberculosis* (MT), particularly in mouse models. MT causes a strong type-1 immune response which does not seem to abrogate EAE in some cases, but may override other possible pathways that drive the disease. Furthermore, some aspects on the protective role of inflammation (Hammarberg H *et al* 2000) could also be explored.

CONCLUDING REMARKS

My studies in this thesis confirm that regions on RN04 and RN15 are linked to MOG-EAE. The QTLs characterized, *Eae19-Eae22* and *Eae24-Eae28,* now have narrow confidence intervals and comprise restricted sets of genes. Interaction analyses revealed interesting features within RN04, such as one QTL that was only detected when epistasis was considered and different sets of QTLs that interact to regulate susceptibility or severity phenotypes. The unbiased identification of rat genes that regulate EAE enables testing of candidate genes association with MS in human studies on large cohorts of patients. Also, for functional studies, congenic rat strains over candidate loci allow gene expression analyses and sequencing and can simplify the molecular studies of pathogenic mechanisms and pathways. Studies of innate immunity aspects of MOG-EAE showed that induction of EAE requires MyD88-dependent signaling pathways but TLR4 and TLR9 regulate rather than promote MOG-EAE. The thesis findings strengthen the genetic and immunologic basis for unraveling disease mechanisms in MS.

ACKNOWLEDGEMENTS

I would like to thank

Tomas Olsson, my main supervisor, because the door to your office is always open for small and big talks, because you fully trust me to be responsible for my work and praise it when it is deserved, for such a varied group of nice "nerds" you assembled and try to shelter from the lack of resources and sexism that abounds in science.

My co-supervisors, Bob Harris, Erik Wallström and Jan Hillert, for being there for me when you were needed.

My collaborators, Anna, for your friendship and so much I learned from you and Johnny, for fruitful writing sessions.

The Neuro group, past and present, for the days, weekends, late evenings when we would have fun around work (Get a life!). In particular, Maja for your friendship and all that you taught me, Pernilla, Åsa, Johan, Ame and Maggan for all the shared knowledge, friendship and support. Kristina, for letting me take over one of your projects and Alan, for keeping on with it. Mohsen, for being the first to teach me in the lab. And Rita, Mélanie, all other former and new students, Brit, Maine, Ami and Mimmi and the people at the AKM department. The VRA and Stem Cells groups, for sharing knowledge and interests. Maria and Rux, you are next! The Rheuma group, in particular Jian for your friendship and great collaboration.

The MS team at Neurology, Tomas, Magnus, Lou, Erik, Fredrik, Agneta, Susanne and Kerstin, for letting me be part of it and reminding me constantly how good it is to be a neurologist.

Tugas e "better-halves" em Stockholm, a Andreia que foi a primeira a receber-me, ao Goncalo, Levi, Ana, Ricardo, Rita e Johan, Sónia e Jack; and other "expatriates", Barbara and Jamie.

Os neurologistas do Hospital de Santo António no Porto, com quem aprendi tanto; especialmente o Dr Luis Monteiro, a Dra Isabel e a Dra Teresa, pelo vosso imenso e constante apoio. Os "internos", pelos dias e noites partilhados que forjaram a nossa amizade. Os outros colegas do hospital, especialmente

os neuroradiologistas, internistas e hematologistas, que partilharam os conhecimentos e companheirismo; o Pedro, pelo apoio e bons conselhos.

As minhas amigas no Porto, Lena and Carla, sempre comigo. My friends from far-away places, Catarina, Ricardo and Ellen, for being close.

Os amigos da Faculdade and Bristol, em particular a Cândida, Bárbara, Guida, Pedro, Mónica and Anja, for sharing good times and bad times.

As amigas de sempre e para sempre, Lisa and Gi, por termos crescido juntas, e sermos adultas (!) com confianca. O Fausto, Álvaro, Pedro, André e todos os amigos de Coimbra.

The Good family for welcoming me into your homes and hearts.

A minha família, do Couto e de Ansião, por todo o carinho e atenções com que me estragam. A avó Augusta, os teus beijinhos estão comigo.

A mãe e o pai, por me terem ensinado que é preferível ser e saber do que ter, por terem lutado por ser e fazer o bem e por gostarem tanto de mim e me terem educado para ser feliz e realizada.

Liam, for who you are and what we are.

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